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Is the Nociception Mechanism Altered in Offspring of Morphine-Abstinent Rats?

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Abstract: **To investigate the effect of parental drug abuse on children, nociception, electrophysiological alteration, mRNA expression of opioid receptors, and expression of certain intracellular proteins in offspring of morphine-abstinent rats were studied. Adult male and female animals received water-soluble morphine for 21 days. Ten days after the last morphine administration, animals were placed for mating in 4 groups as follows: healthy (drug naive) female and male, morphine-abstinent female and healthy male, morphine-abstinent male and healthy female, morphine-abstinent male and morphine-abstinent female. Their adult male offspring were tested for nociception, neuronal discharge in nucleus accumbens (NAC) and prefrontal cortex (PFC). Our results showed that nociception in male offspring of all morphine-abstinent parent(s) groups was significantly reduced, compared with the control group. In the offspring of morphine-abstinent parent(s) groups, sensitivity to the antinociceptive effect of morphine was enhanced in chronic as well as in acute phases of the formalin test. Neuronal electrical activity reduced in the offspring of the morphine-exposed parent(s) in NAC as well as PFC regions. Moreover, our findings show that opioid receptors' expressions (μ, κ, and δ) increased in NAC of the litter of morphine-abstinent parent(s), compared with the control group. In addition, the expression of κ receptors was remarkably increased in the PFC in morphine-abstinent parent group, relative to the control group. The phosphorylated levels of extracellular regulated kinase 1/2 and cyclic adenosine monophosphate responsive element binding protein were significantly higher in the offspring of the morphine-abstinent parent(s) than the control group in the NAC. Our results indicated that endogenous opioid is altered in offspring of the morphine-exposed parent(s) and that heritage has a major role.**

Perspective: ***This study showed that nociception was reduced in offspring of morphine-abstinent rat(s) and also these litters had a low level of neuronal firing rate, and enhanced opioid receptors expression, especially in the NAC. Because these offspring are more sensitive to the analgesic effect of morphine, clinicians should consider this issue to manage the dosage of morphine for treating pain in children with an abstinent parent(s).***

Key words: *Morphine, pain, cyclic adenosine monophosphate responsive element binding, electrophysiology, opioid receptors expression.*

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Family, twin, and adoption studies have yielded enormous amounts of valuable information about drug dependence as a multifactorial and polygenic disorder.[30,32] Chronic drug exposure renders lasting molecular and cellular changes in the central nervous system including alternations in gene expression, mRNA level, protein level, and synaptic plasticity.[39] Molecular as well as behavioral studies indicate that addictive substances induce long-lasting changes in the behavior and gene expression in some brain regions involved in reward circuit.[21,31,61] These features of drug abuse could initiate epigenetic mechanisms and some epigenetic factors such as DNA methylation, histone acetylation, and micro RNA production, contributing to inheritance of drug addiction.[4,13] Epigenetic transmission is conceptualized as alternations in gene expression (not DNA sequence) in consequences of parental behavior and environmental conditions, which might alter vulnerability to develop an addictive phenotype.[8,17,60] Additionally, oocyte as well as sperm possess opioid receptors; therefore, alterations in opioid receptors expression could transfer to the next generation,[1] and that is why offspring of addicted parents are subjected to a greater vulnerability to psychiatric disorders, drug abuse, and social abnormalities.[38,40]

The opioid system comprises 3 types of receptors (μ, κ, and δ receptors) and is a master key in the control of different physiological activities including mood, learning, and memory.[47] Likewise, the opioidergic system regulates the sensitivity to noxious stimuli.[67] Morphine—one of the alkaloids of opium—has a common clinical use despite its several side effects[50] and regulates the intracellular processing of nociceptive mechanisms.[44]

Noxious stimuli engender pain, after tissue damage, which is an unpleasant sensory experience. One of the physiological functions of pain is motivation to push back from damaging stimuli to protect the body.[7,11] The sensory experience of pain is highly related to emotional state, past experiences, memories, etc.[41] To date, morphine is the most efficient drug for musculoskeletal and surgical pain suppression.[28] Morphine administration stimulates all 3 types of opioid receptors yielding pain relief through different cellular mechanisms.[46] However, there is no evidence on the role of parental morphine dependence on the behavioral and molecular changes in the offspring. However, one study has reported that male and female offspring of morphine-dependent parents had impairment in memory formation and long-term potentiation.[49]

Up to now, there have been only a limited number of studies about the long-term consequences of pain alterations in morphine-abstinent parents' offspring. In this study, we used an animal model to examine the nociception threshold in the offspring of the morphine-abstinent parent(s) to investigate the possible paternal and/or maternal contribution to the epigenetic inheritance of nociception profiles. We evaluated opioid receptors gene expression (μ, δ, and κ), extracellular neuronal discharge and the level of μ opioid receptor, extracellular regulated kinase (ERK), and cyclic adenosine monophosphate responsive element binding (CREB) phosphorylation state (the downstream proteins of opioid receptors in the brain) in the nucleus accumbens (NAC) and prefrontal cortex (PFC) in the litters of morphine-abstinent parent(s).

Methods

Animals

Adult male and female Wistar rats (weight 220–250 g for male and 180–220 g for female, 10-week-old) were used in this study. The animals were housed 4 rats per Plexiglas cage and maintained in a room with controlled light/dark cycle (12/12 hours with light beginning at 7:00 AM) and temperature (22°C ± 2°C), with free access to food and water. All procedures in this investigation were approved by the Tehran University of Medical Sciences' Ethics Committee, which corresponds to the national guidelines for animal care (National Institutes of Health guidelines; Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 86-23).

Experimental Procedure

Parental Morphine Exposure

Twenty male and 20 female rats were treated with oral morphine sulfate (Temad Co, Tehran, Iran) for 21 days.[3] The morphine was administrated orally in doses of .1 mg/mL (day 1 and 2), .2 mg/mL (day 3 and 4), and .3 mg/mL (day 5 and 6), and then .4 mg/mL for the remaining 15 days. Sucrose (3%; Merck, Darmstadt, Germany) was added to counter the bitter taste of morphine. Twenty male and 20 female rats received only sucrose (3%) in their water as a vehicle. After 21 days, dependence was proven by naloxone (a μ-opioid receptor antagonist; Sigma, St. Louis, MO) injection (intraperitoneal [I.P.]) at the dose of 10 mg/kg for all rats. Withdrawal symptoms such as wet dog shake, diarrhea, yawning, teeth chattering, swiping tail movement, and time of ptosis were recorded 5 minutes after naloxone injection.

Mating Protocol

Ten days after the last morphine administration, abstinent and healthy animals were randomly assigned into 4 groups for mating, as follows: 10 healthy (drug-naive) female and 10 healthy male rats (litters of this group named as the control group), 10 morphine-abstinent female and 10 drug-naive male rats (litters of this group named as maternal morphine-exposed [M.ME]), 10 morphine-abstinent male and 10 drug-naive female rats (litters of this group named as the paternal morphine-exposed [P.ME] group), 10 morphine-abstinent male and 10 morphine-abstinent female rats (litters of this group named as the M.ME+P.ME group). One female and 1 male rat were put in a cage and were monitored daily for vaginal plaque. The number of births and deaths of litters was recorded. We used male offspring of the first parturition (Fig 1). One hundred ninety-two male offspring of morphine-abstinent or healthy parent(s) (2 months) were randomly divided into 6 subgroups. Behavioral tests included median effective dose 50 (ED_{50}) of morphine in

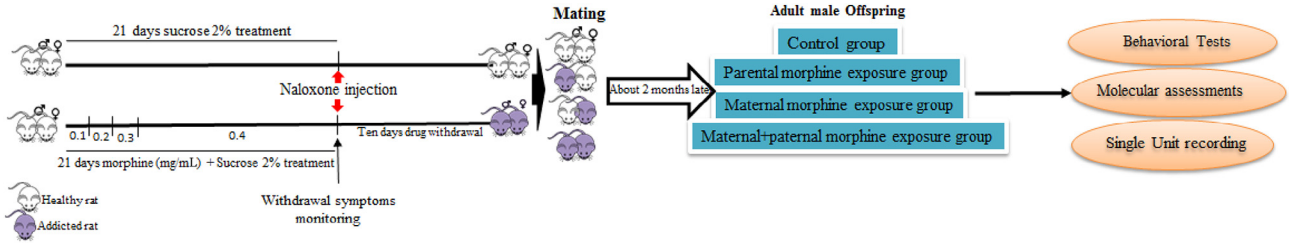

Figure 1. Timeline schedule of experimental design.

formalin test, formalin nociception, morphine analgesic effect in formalin test, writhing and hot plate tests (n = 8 per group; 160 rats were used), molecular (n = 8 per group) and electrophysiological assessments (n = 8 per group; 32 rats were used). Each rat was used once for each experiment. For determining the dose response curve in formalin test, we used 100 adult male drug-naive rats.

Hot Plate Test

Acute thermal pain threshold in offspring was determined using the hot plate test. This test was performed by placing a rat on a 52°C plate (Borj Sanaat, Tehran, Iran) and measuring the thermal withdrawal latency. A cutoff period (30 seconds) was used to prevent tissue damage.[57]

Writhing Test

The writhing test is a chemical method. In this method, injection of acetic acid (Merck) into the peritoneum induces visceral pain. Briefly, all animals were placed in a small observation chamber. After 10 minutes of habituation, rats received acetic acid (.8%) in a volume of 10 mL/kg into the peritoneum. Nociceptive behavior was characterized by abdominal contraction known as writhing, which is described as an exaggerated extension of the abdomen combined with the outstretching of hind limbs. Five minutes after the I.P. administration of acetic acid, the number of writhing and total time of writhing were recorded for over 10 minutes.[52]

Formalin Test

The formalin (Merck) test is applied to the study of acute and chronic pain. Briefly, the pain is induced by applying .1 mL of 2.5% formalin into the dorsal surface of the left hind paw of each rat. The rats were placed in an observation chamber with a mirror mounted on 3 sides to allow a clear view of the paws. The time each rat spent licking the injected paws (licking time) was recorded. Acute pain, essentially resulting from the direct stimulation of nociceptors, was observed in 1- to 10-minute intervals after the formalin injection, whereas chronic pain, involving a period of sensitization during which inflammatory phenomena, was observed in 20- to 40-minute intervals, after the formalin injection.[15,27]

To evaluate the contribution of the opioidergic system in offspring of the morphine-abstinent parent(s), a pretreatment with naloxone at 10 mg/kg, I.P. 15 minutes before the formalin test was performed.[23,36] The animals were subjected to the naloxone treatment before the formalin test.

Extracellular Single-Unit Recording

A perylene-coated tungsten microelectrode (WPI, Sarasota, FL; with extra-fine tip; 1 MΩ impedance tip) was inserted stereotactically into the NAC (anterior-posterior = 1 mm, mediolateral = 1 mm, Dorsoventral = 5.5 mm) and PFC (anterior-posterior = 3.2 mm, mediolateral = .75 mm, Dorsoventral = 6.7 mm) of the left side of the brain.[42] Then, the electrode was guided into the NAC and/or PFC using a manual microdrive, until maximum spike activity was detected with a signal-to-noise ratio of >2, isolated from the background noise. Signals from the electrode were preamplified for impedance matching with a unity gain preamplifier, amplified 10,000 times using a differential amplifier (Electromodule R12, Science- Beam, Tehran, Iran), band-pass filtered at .3 to 10 kHz, and digitalized at 50 kHz sampling rate and 12-bit voltage resolution, using a data acquisition system (Electromodule R12, Science- Beam, Tehran, Iran). All-or-none spike events were detected using a window discriminator (Electromodule R12, Science- Beam, Tehran, Iran) on the basis of the spike amplitude. The spike frequencies were counted and displayed online in time bins of 1,000 ms over the entire recording period by online-sorter software (eprobe.1.42, Science-Beam, Tehran, Iran).[18] Clusters were formed by spikes of individual neurons tracked. We traced 22.0 ± 3 neurons in the NAC and 34.2 ± 4 types of neurons in PFC. Spikes of single units were quarantined with a signal-to-noise ratio of ≥3:1.[25,48] The excitatory spikes were counted per time bin and evaluated as firing rate. When NAC and/or PFC neurons with steady firing rate were noticed in actual firing rate for 10 minutes, and the recording was carried on for approximately 20 minutes. The sites of neuronal recording (in the NAC and PFC) were verified according to the Paxinos and Watson Atlas,[42] and a separate group of abstinent parents' offspring were used for electrophysiological studies.

Brain Tissue Collection

For evaluating the opioid receptor mRNA expression pattern in offspring of the morphine-exposed parent(s),

5 male rats in each group were sacrificed; the PFC and NAC were dissected and immediately frozen in liquid nitrogen and kept in –80°C for Western blot analysis and real-time polymerase chain reaction (PCR) methods.

Real-Time PCR

Total RNA was extracted from the PFC and NAC with RNeasy lipid tissue mini kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. With a spectrophotometer, we determined the RNA quantity and purity. Also, agarose gel electrophoresis was performed to check RNA integrity (.8% agarose; Gibco/BRL, Indianapolis, IN). cDNA synthesis was carried out with Prime Script First-Strand (cDNA Synthesis Kit; Takara, Takara, Japan). Total RNA (1 μg) was reverse transcripted to complementary DNA in a final volume of 20 μL. All primers (μ, δ, and κ opioid receptors and β-actin as an endogenous control gene) were purchased from Qiagen primer bank. We used a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) for real-time PCR reaction. cDNA (2 μL), 2 μL of primer, and SYBR Green Master Mix (Takara) were mixed according to manufacturer protocol in a total volume of 20 μL. The annealing temperature of μ and κ opioid receptors was adjusted to 60°C. δ Opioid receptor annealing temperature was set to 58°C. The specificities of PCR products for each gene were confirmed by a single peak in melt curve and visualization in 2% agarose gel.

Western Blot Technique

Western blot analysis was performed to determine protein levels in the PFC and the NAC. Proteins of both regions were extracted in radioimmunoprecipitation assay buffer. A total of 60 μg of proteins were electrophoresed on 12.5% gradient gels (Bio-Rad Laboratories, Hercules, CA). The proteins were transferred to polyvinylidene fluoride membranes. Then, the membranes were incubated with primary antibodies including phosphorylated CREB, total CREB, phosphorylated ERK1/2, total ERK1/2 and μ opioid receptor (1/1,000; Cell Signaling Technology Co, Danvers, MA). The membranes were incubated with the secondary antibody (1/5,000; Cell Signaling Technology Co) and blots were developed using the ECL Advanced Kit (Amersham Bioscience Co, Piscataway, NJ). The polyvinylidene fluoride membranes were stripped and reused using antiactin antibody (1/1,000; Cell Signaling Technology Co) to normalize protein loading and transfer. Protein bands were detected on X-ray radiology film. Image J software (Version 1.41, National Institutes of Health, Bethesda, MD) was used for densitometry analysis and quantification of results.

Statistical Analysis

Obtained data were expressed as mean ± standard error of the mean. Behavioral (n = 8 per group), electrophysiological (n = 8 per group) and Western blot (n = 5 per group) results were analyzed statistically using 1-way analysis of variance followed by Tukey multiple comparison tests using SPSS version 21 software (IBM Corp, Armonk, NY). Gene expression data (n = 5 per group) were analyzed using Relative Expression Software Tool (REST)-XL version 2.[43] This software determines differences in relative gene expression level of the samples compared with the control group. The ED_{50} value for each group was determined using linear regression, with SPSS version 21 software (IBM Corp; n = 8 per group). The ED_{50} values were measured as means attended by 95% confidence limits.

Results

Parental Morphine Administration Before Mating Increased Offspring's Mortality

Total mortality of pups was recorded for each group. Table 1 shows the mortality rate among morphine-abstinence-derived offspring and control. As shown in Table 1, χ^2 analysis showed that mortality rate had a significant increase in groups that had 1 and/or 2 morphine-abstinent parent(s) ($\chi^2 = 9.31$, $P = .02$). The number of pups in each litter was not changed among groups ($P = .25$).

Naloxone Increased the Withdrawal Symptoms in Male as Well as Female Morphine-Dependent Parents

As shown in Fig 2, all measured withdrawal symptoms (wet dog shake, yawning, diarrhea, tail movement, teeth chattering, and time of ptosis) increased in morphine-exposed rats, compared with vehicle-exposed rats, in both sexes ($P < .001$).

Thermal and Visceral Nociception Decreased in the Offspring of Morphine-Abstinent Parent(s)

The hotplate test was used to evaluate pain reflexes in response to thermal stimulus. Fig 3A shows the effect of maternal and/or paternal morphine exposure in the hot plate test. As shown in Fig 3A, nociception was reduced in groups with 1 and/or 2 morphine-abstinent parent(s) compared with drug-naive parents ($F_{3,28} = 6.97$, $P < .01$). The writhing test was used to

Table 1. Analysis of Parental Morphine Administration Before Mating on the Total Mortality Rate of Offspring and Number of Pups in Each Litter in Experimental Groups

Group	Total Mortality Rate, %	Number of Litters in Each Parturition
Control	6.09 ± 2.3	6.66 ± 1.2
M.ME	17.74 ± 7.6	5.66 ± 1.0
P.ME	12.00 ± 7.5	7.16 ± 1.3
M.ME+P.ME	20.73 ± 8.1	6.33 ± 1.5

NOTE. Data are represented as mean ± standard error of the mean.

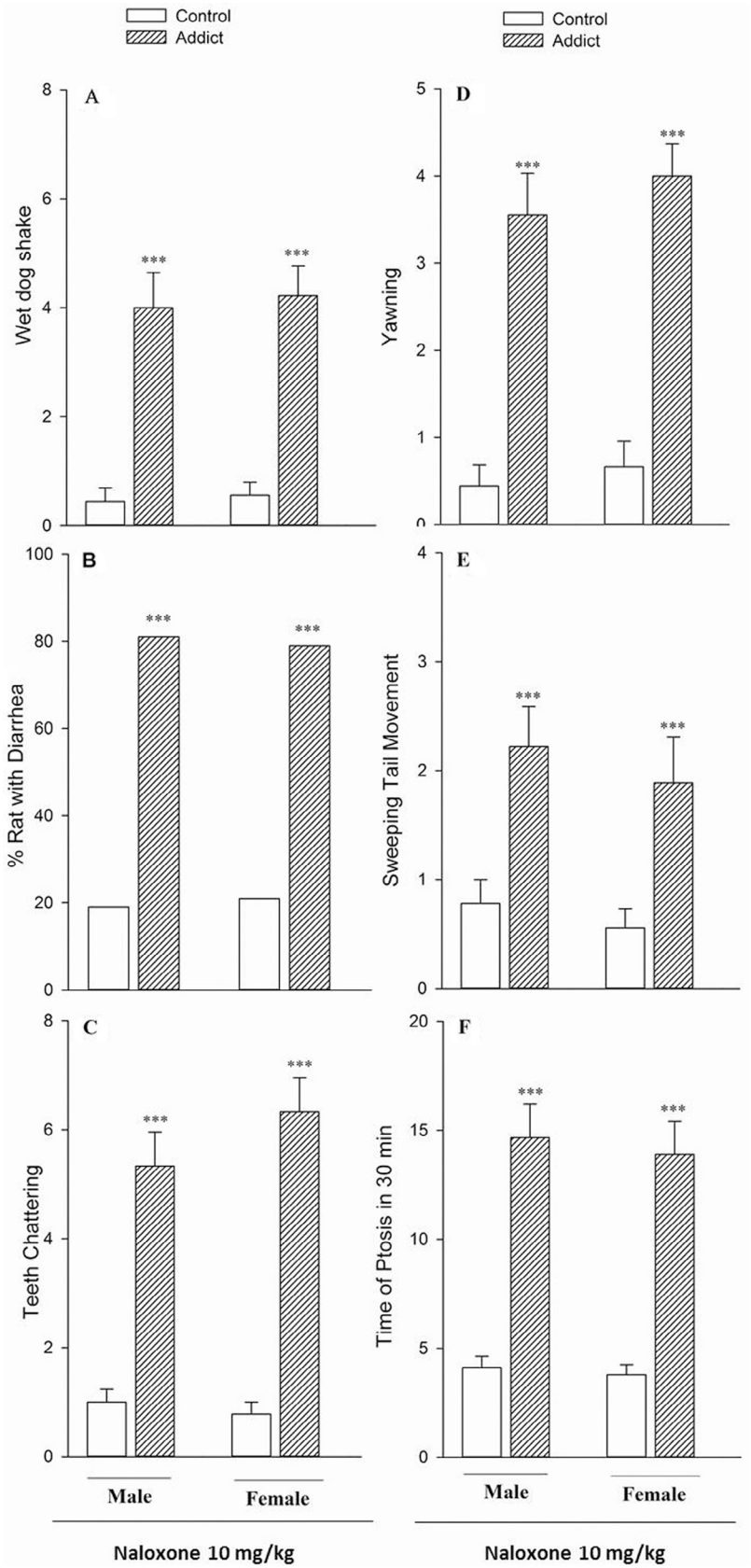

Figure 2. Effect of naloxone treatment in control and addict parents. (**A**) Wet dog shake, (**B**) percentage of diarrhea, (**C**) teeth chattering, (**D**) yawing, (**E**) stretching tail movement, and (**F**) time of ptosis in 30 minutes. Values are the mean ± standard error of the mean for 8 rats in control, M.ME, P.ME, and M.ME+P.ME groups. ***$P < .001$ versus its control group.

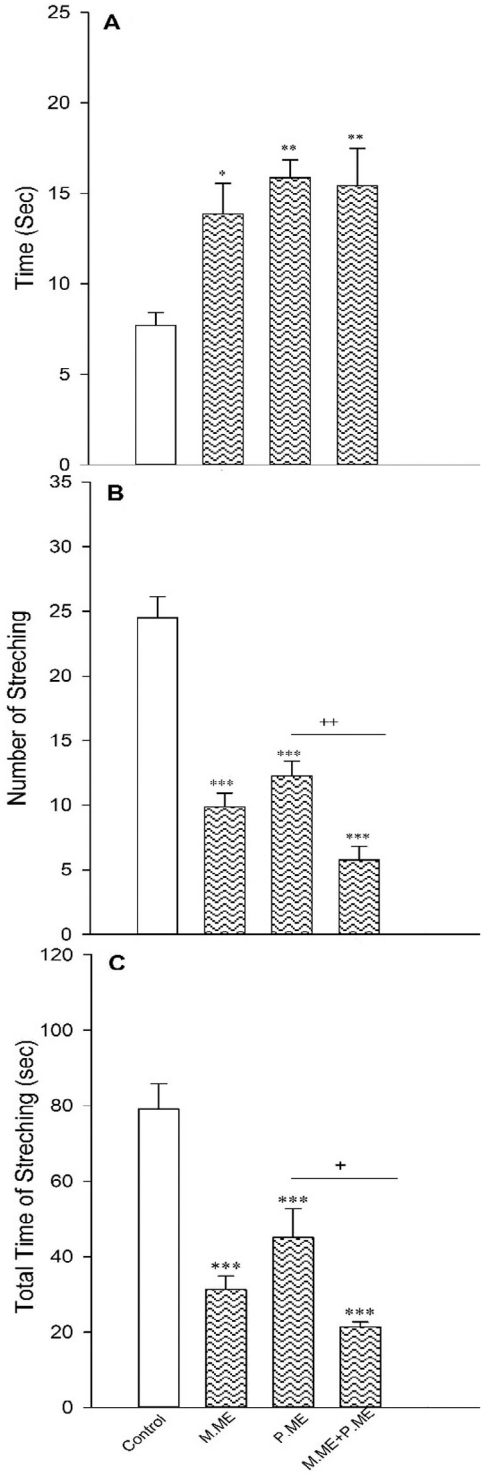

Figure 3. Nociception tests. (**A**) Thermal pain reflexes measured in experimental groups. (**B**) Total time of stretching and (**C**) the number of stretching in morphine-abstinent-derived offspring in the acetic acid-induced writhing test. Values are the mean ± standard error of the mean for 8 rats in control, M.ME, P.ME and M.ME+P.ME groups. *$P < .05$, **$P < .01$, ***$P < .001$ versus control group. +$P < .05$, ++$P < .01$ versus P.ME.

measure visceral pain.[45] The effect of parental morphine exposure before mating, on acetic acid-induced writhing, is shown in Fig 3B. The total number of stretching during 10 minutes was decreased in groups with 1 and/or 2 morphine-abstinent parent(s) ($F_{3,28} = 41.40$, $P < .001$). Fig 3C shows that total time of stretching was decreased in rats with 1 and/or 2 morphine-abstinent parent(s), compared with the control group ($F_{3,28} = 21.55$, $P < .001$). The total time and number of stretching increased in the offspring of both abstinent parents (M.ME+P.ME group) compared with the offspring of an abstinent father and healthy mother (P.ME group).

Parental Morphine Administration Before Mating Decreased Formalin-Induced Nociception in Offspring

Administration of formalin as noxious stimuli in hind paw-induced pain in the control rats. Fig 4 shows the effect of parental morphine exposure before gestation on the (Fig 4A) acute pain (early phase) and (Fig 4B) chronic pain (late phase) in the formalin test with or without administering naloxone injection in male offspring. Fig 4A shows that the total time of paw-licking in acute pain (first 10 minutes in formalin test) was decreased in offspring with 1 and/or 2 morphine-abstinent parent(s) ($F_{3,28} = 11.64$, $P < .001$). In addition, in the late phase of formalin-induced pain (Fig 4B), a significant reduction of nociception in the offspring of both morphine-abstinent parents was observed ($F_{3,28} = 6.01$, $P = .003$). Naloxone was injected before the formalin test to confirm the increased endogenous opioid in offspring of morphine-abstinent rats. Data supported that naloxone administration significantly increased acute and chronic nociception in the formalin test in M.ME, P.ME, and M.ME+P.ME groups, compared with the control group ($P < .001$; Figs 4A and 4B).

Morphine-Abstinence-Derived Offspring Showed Significantly Enhanced Sensitivity to Antinociceptive Effect of Morphine in Formalin Test

Fig 4C shows the antinociceptive effect of different doses of morphine (1.5, 3, and 6 mg/kg, subcutaneous [s.c.]) on pain induced by formalin in the offspring of healthy parents. Administration of morphine (3 and 6 mg/kg, s.c.) 15 minutes before the formalin test led to decreased nociception compared with the saline-treated group. This effect of opioid was apparent in acute ($F_{3,28} = 33.85$, $P < .001$) as well as chronic ($F_{3,28} = 56.75$, $P < .001$) phases of the formalin test (Fig 4C). Because of these results, a noneffective dose of morphine (1.5 mg/kg, s.c.) was used for subsequent studies.

Furthermore, we determined ED_{50} values for all the experimental groups in chronic as well as acute phases (Table 2). Significant changes are shown in Table 2 in P.ME, M.ME, and P.ME+M.ME groups compared with the control group ($P < .01$, $P < .01$, and $P < .001$, in both phases, respectively). Administration of a noneffective dose of morphine (1.5 mg/kg s.c.) gave rise to decreased

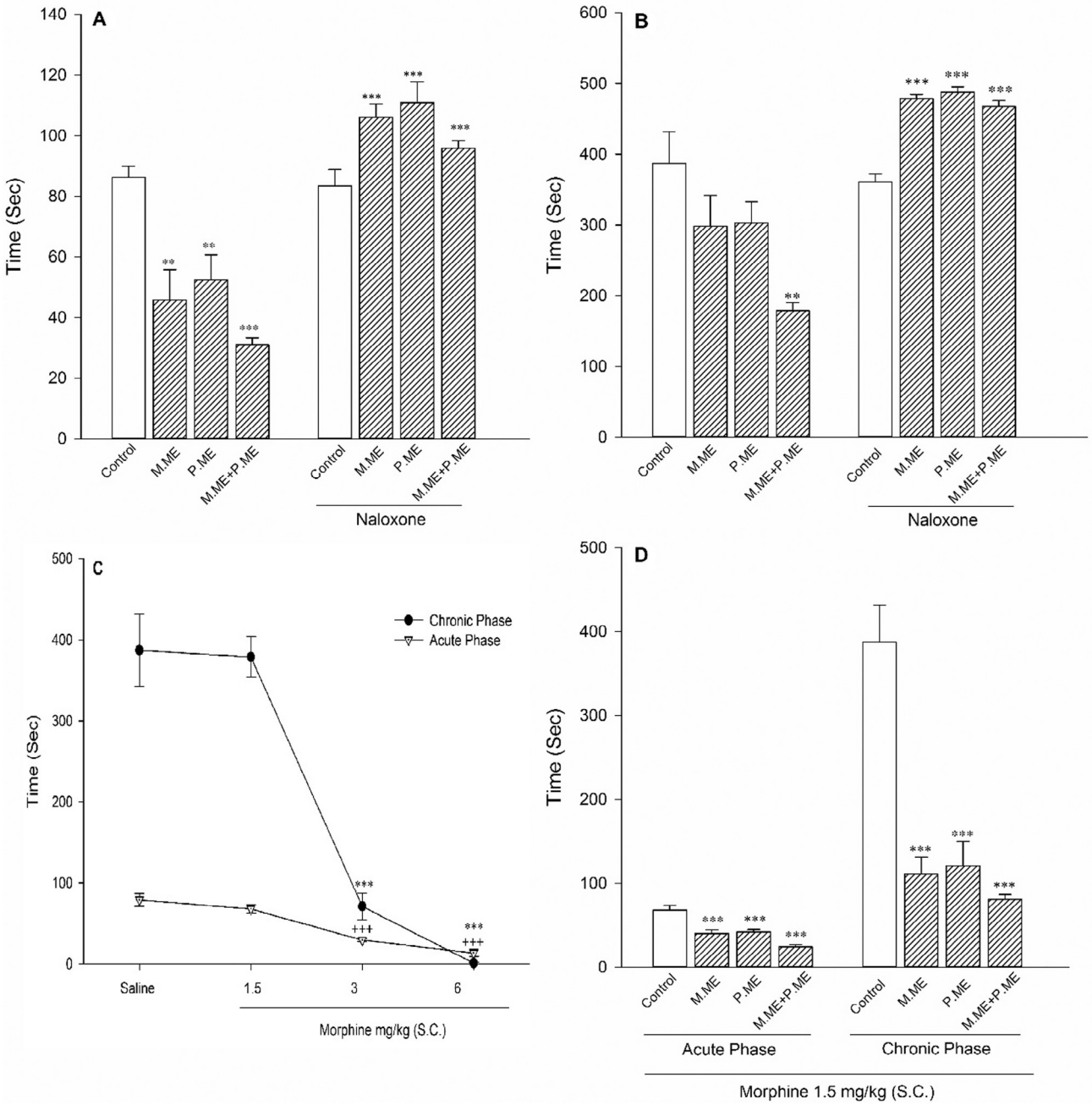

Figure 4. Formalin test. (**A**) Acute and (**B**) chronic formalin-induced pain test and in the presence of naloxone in control, M.ME, P.ME, and M.ME+P.ME groups. ***$P < .001$ versus control group in the acute phase. +++$P < .001$ versus control group in acute phase. (**C**) Total time of paw-licking in acute and chronic phases of the formalin test in control rats, which received 1.5, 3, and 6 mg/kg morphine. (**D**) Effect of morphine (1.5 mg/kg) injection on the total time of paw-licking in acute and chronic phases in the formalin test of offspring in control, M.ME, P.ME, and M.ME+P.ME groups (n = 8 for each group). Values are presented as the mean ± standard error of the mean for 8 rats in each group. **$P < .01$, ***$P < .001$ versus the control group.

Table 2. Comparison of Morphine ED_{50} (mg/kg. I.P.; With 95% Confidence Limits) for Acute and Chronic Pain in the Formalin Test

Group	*Acute Phase*	*Chronic Phase*
Control	2.31 ± .05	1.97 ± .06
P.ME	2.51 ± .06**	2.23 ± .05**
M.ME	2.56 ± .06**	2.25 ± .07**
P.ME+M.ME	2.84 ± .07***	2.60 ± .07***

NOTE. Data are represented as mean ± standard error of the mean.
**$P < .01$ versus control, Dunnett t-test.
***$P < .001$ versus control, Dunnett t-test.

nociception in offspring of the morphine-abstinent parent(s). Fig 4D shows the antinociceptive effect of a low dose of morphine in acute and chronic pain. The litter of morphine-abstinent parent(s) was more sensitive to the antinociceptive effect of morphine, compared with the control group ($F_{3,28} = 19.31$, $P < .001$). Also, a noneffective dose of morphine (1.5 mg/kg) had an antinociceptive activity in the late phase of formalin test in offspring of the morphine-abstinent parents ($F_{3,28} = 25.07$, $P < .001$; Fig 4D).

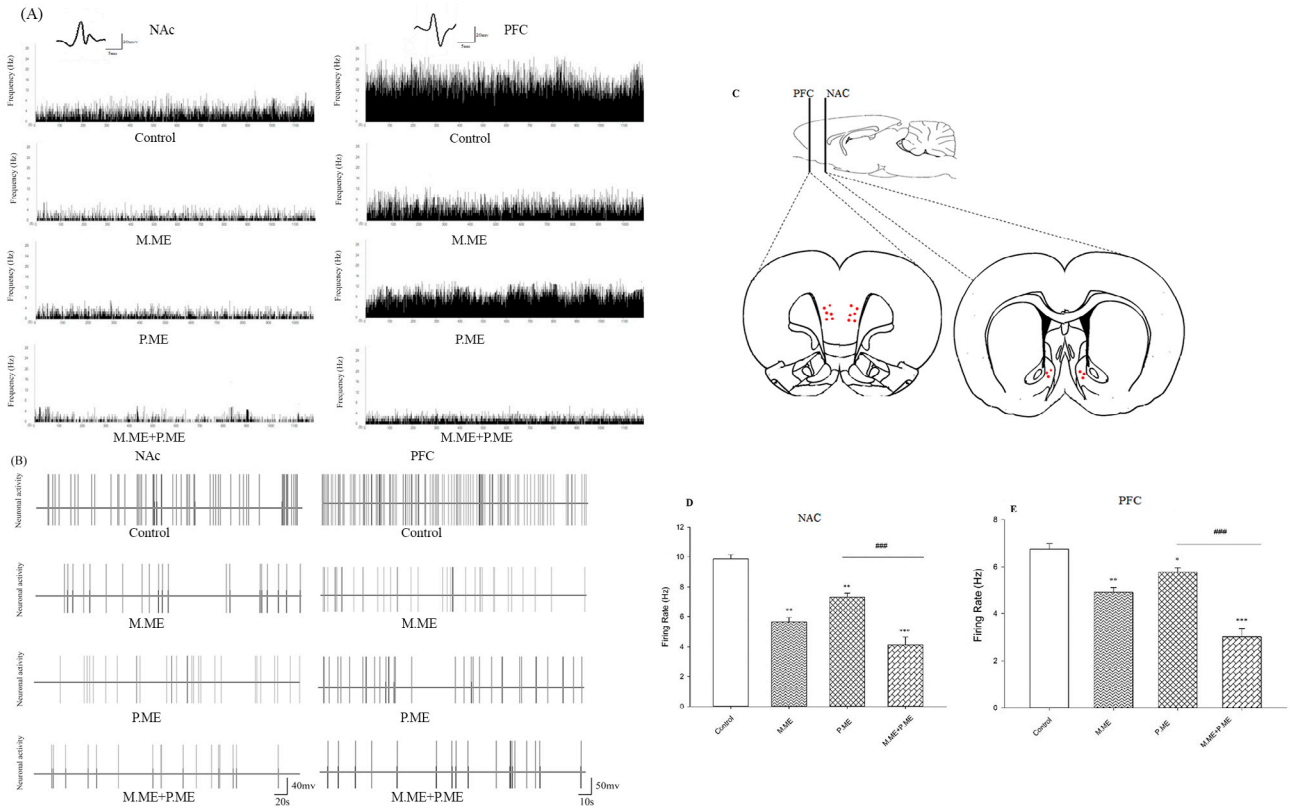

Figure 5. The frequency of spikes in the NAC and PFC in control, M.ME, P.ME, and M.ME+P.ME groups. (**A**) The left column shows the frequency of spikes in the NAC and a sample spike of the NAC neurons indicated at the top of the left column. (**B**) The right column shows the frequency of spikes in the PFC and a sample spike of the PFC neurons indicated at the top of the right column. (**C**) Red dots show the sites of recording in the NAC and PFC. (**D**) The quantitative firing rate of the NAC neurons (mean of recorded neurons number = 22.00). (**E**) The quantitative firing rate of the PFC neurons (mean of recorded neurons number = 34.20). Data were normalized on the basis of the control group and percentage of changes shown. Values are presented as mean ± standard error of the mean; n = 8. **P* < .05, ***P* < .01, ****P* < .001 versus control group, ###*P* < .001 versus P.ME.

Parental Morphine Exposure Before Mating Reduced Offspring's Spontaneous Neuronal Activity in the NAC and PFC

As shown in Fig 5A, the frequency of spikes in the NAC and PFC regions were detected. The left column in Fig 5A shows the frequency of spikes in the NAC and an action potential presented in this region. The right column in Fig 5A indicates the frequency of spikes in the PFC area of the brain, and also an action potential was shown in this region. Fig 5B indicates the neuronal activity in the NAC (left column) and PFC (right column) in the 4 experimental groups (control, M.ME, P.ME, and M.ME+P.ME groups). Fig 5C shows the sites of records in the PFC and NAC (red dots). The quantitative analysis of neuronal discharging in the NAC and PFC measured is shown in Figs 5D and 5E. Results indicated that neuronal firing activity in the NAC was reduced in the M.ME ($P < .001$), P.ME ($P < .01$), and M.ME+P.ME ($P < .001$) groups compared with the control group ($F_{3,28} = 85.05$, $P < .001$.) Similarly, firing rate in the PFC area attenuated in the M.ME ($P < .01$), P.ME ($P < .05$), and M.ME+P.ME ($P < .001$) groups compared with the control group (Fig 5E; $F_{3,28} = 19.93$, $P < .001$). Neuronal firing activity was increased in the M.ME+P.ME group compared with the P.ME group in NAC as well as PFC regions ($P < .001$).

The mRNA Level of Opioid Receptors Changed in the PFC and NAC in the Litter of Morphine-Abstinent Parents

The mRNA level of μ, κ, and δ opioid receptors in the NAC and PFC are shown in Fig 6. Figs 6A, 6B, and 6C shows the mRNA expression level of 3 opioid receptors (μ, κ, and δ) in the NAC of the litter of morphine-abstinent rats. In all 3 groups (M.ME, P.ME, and M.ME+P.ME), the μ opioid receptor was upregulated by the factors 19.19 ($P < .001$), 7.38 ($P < .001$), and 4.604 ($P < .001$), respectively, compared with the controls (Fig 6A). The expression level of the κ opioid receptor mRNA increased in M.ME, P.ME, and M.ME+P.ME groups by the factors 6.071 ($P < .001$), 1.54 ($P = .038$), and 1.815 ($P < .001$) in that order (Fig 6B). The δ opioid receptor was upregulated in M.ME ($P = .02$), P.ME ($P < .001$), and M.ME+P.ME ($P = .04$) compared with the control group (Fig 6C). The level of μ opioid receptor mRNA in the PFC of morphine-abstinent rats' offspring did not change compared with the control group (Fig 6D). Fig 6E shows that the κ opioid receptor mRNA level was enhanced in groups that had 1 morphine-abstinent parent

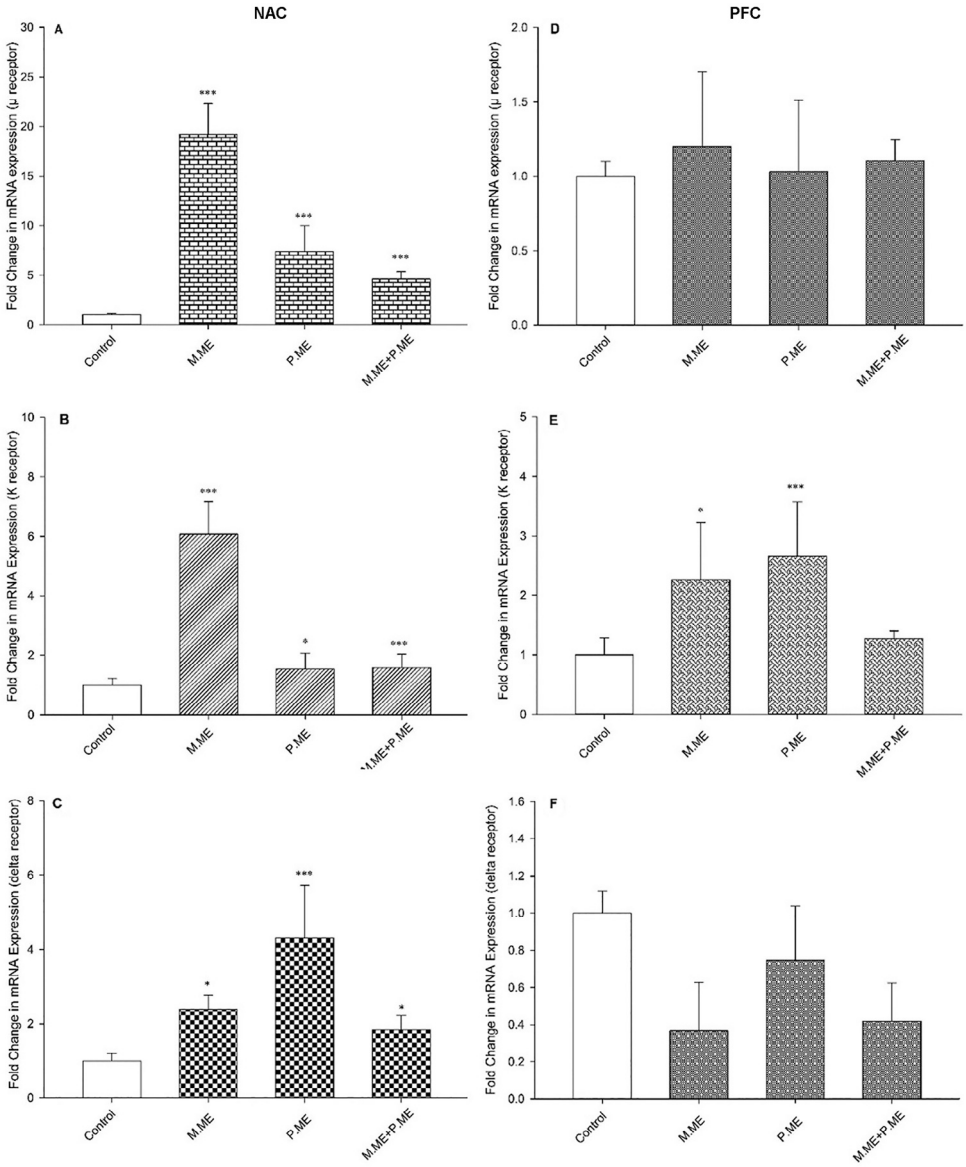

Figure 6. The mRNA expression level of (**A**) μ, (**B**) κ, and (**C**) δ opioid receptors in the PFC and (**D**) μ, (**E**) κ, and (**F**) δ opioid receptors in the NAC of control, M.ME, P.ME and M.ME+P.ME groups. Bars represent fold differences of mean normalized expression value ± standard error of the mean (n = 5). *$P < .05$ and ***$P < .001$ versus control group. All data were compared with the control group.

compared with the control group ($P = .02$ and $P < .001$ for M.ME and P.ME, respectively). However, the δ opioid receptor was downregulated in the offspring of morphine-abstinent rats, but, it was not statistically significant ($P = .06$, $P = .28$, and $P = .69$ for M.ME, P.ME, and M.ME+P.ME respectively; Fig 6C).

Mu Opioid Receptors, CREB, and ERK1/2 Phosphorylation Increased in the NAC and PFC of Morphine-Exposed Parent(s) Offspring

The changes of the μ opioid receptor level, CREB, and ERK1/2 phosphorylation in the NAC and PFC is shown in Fig 7A. The Western blot technique showed that the protein level of μ opioid receptors; phosphorylated CREB, and ERK1/2 were significantly increased in the NAC (Figs 7B–D) of the morphine-abstinent parents' offspring compared with the control group. The ratio of μ opioid receptor ($F_{3,16} = 17.95$, $P < .001$) and phosphorylated CREB to total CREB ($F_{3,16} = 44.62$, $P < .001$) increased in the M.ME, P.ME, and M.ME+P.ME groups compared with the control group in NAC ($P < .001$, Fig 7B and 7C). Similarly, the phosphorylated ERK1/2 to total ERK1/2 ratio ($F_{3,16} = 76.04$, $P < .001$) was increased in the M.ME, P.ME, and M.ME+P.ME groups compared with the control group in NAC ($P < .001$, Fig 7D). There were no statistically significant changes in μ opioid receptors, ERK1/2, and CREB phosphorylation among groups in the PFC ($P > .05$, Figs 7E–G).

Discussion

Previous studies indicated that maternal morphine exposure induced sensitization to morphine in male as well as female offspring.[5] Moreover, it has been shown that offspring of addicted fathers have some congenital prob-

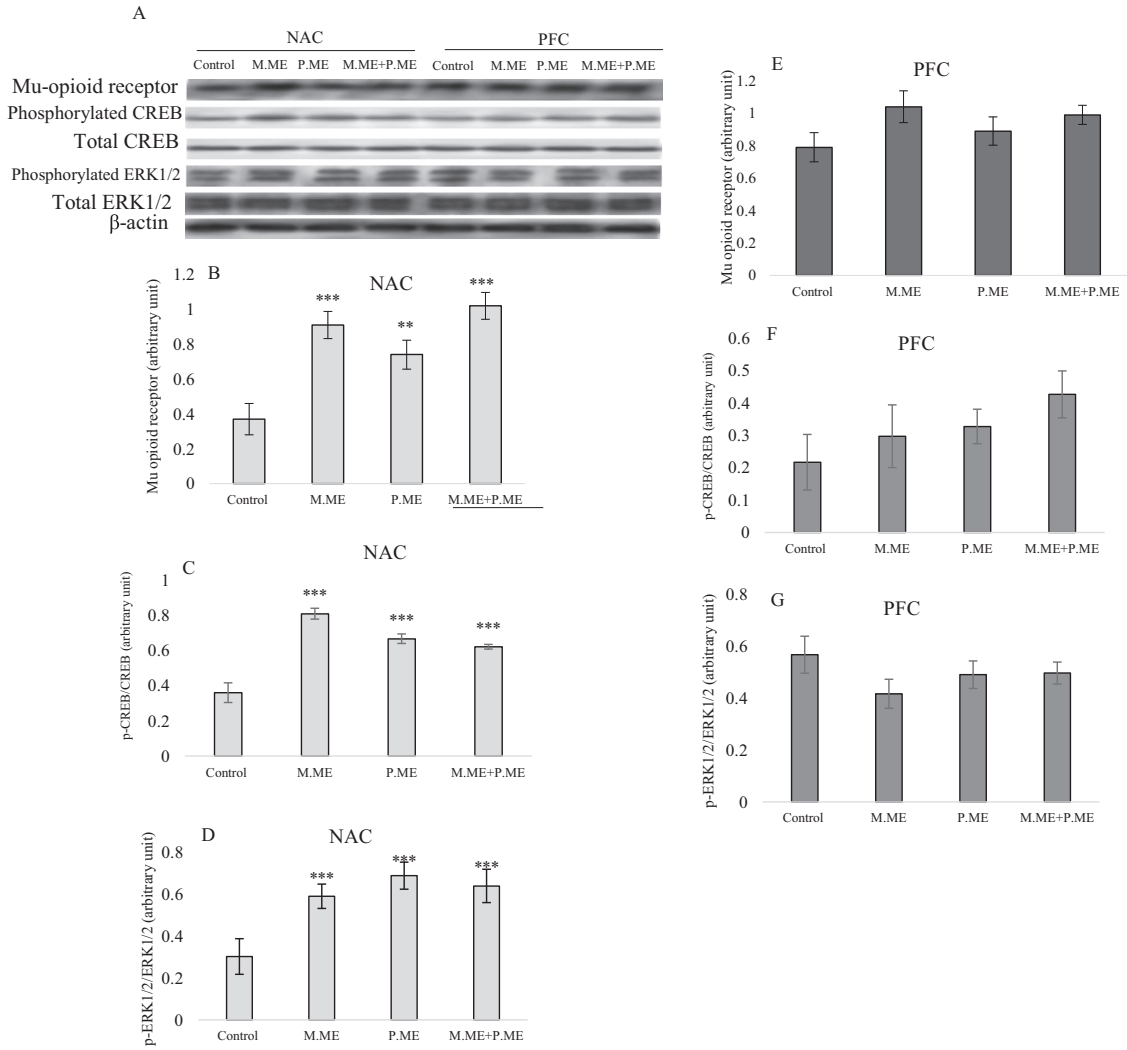

Figure 7. The protein level of μ opioid receptor, phosphorylated CREB, phosphorylated ERK1/2 in the PFC and NAC (**A**) in control, M.ME, P.ME, and M.ME+P.ME groups. The density of μ opioid receptor/β-actin (**B**), phosphorylated (p-)CREB/total CREB (**C**) and p-ERK/total ERK (**D**) in the NAC were measured using Image J software (National Institutes of Health, Bethesda, MD). The density of μ opioid receptor/β-actin (**E**), p-CREB/total CREB (**F**), and p-ERK/total ERK (**G**) in the PFC were measured using Image J, version 1.41 software. Bars represent mean ± standard error of the mean (n = 5). ***$P < .001$ versus control group.

lems and developmental impairments including weight loss, behavioral abnormalities, learning, and memory deficit.[49,63] In sperm of men who suffered from opioid addiction, opioid receptors genes methylated through DNA methyltransferase, and, subsequently, this hypermethylation was inherited to the next generation.[9]

Herein, we claim that chronic, acute, thermal, and visceral nociception were decreased in the litters of 1 and/or 2 morphine-abstinent rat(s). In keeping with our findings, Byrnes and colleagues reported that male offspring of morphine-dependent mothers had an increased level of sensitivity to acute morphine analgesic effects and enhanced morphine tolerance.[6] Our results contradict findings of Chorbov et al, who declared the offspring of addicted parents had no analgesic effect in nociception.[9] Moreover, our study showed that male offspring of morphine-abstinent rats have more analgesic response to a noneffective dose of morphine in the late phase of the formalin test. Hypermethylated DNA was bequeathed to the offspring and caused a reduction in opioid receptor mu 1 (*OPRM1*) gene transcription; which proposed that *OPRM1* transcription reduced nociception in offspring of addicted fathers.[9] Hypermethylation is not the sole reason for hyposensitivity to noxious stimuli in the next generation of morphine-dependent parents. Pain sensitivity might relate perceiving intensity of painful stimuli or response to antinociceptive effects of morphine characterized by environmental conditions, genetic factors, and history of parental morphine exposure.[16]

Pain experience reduces midbrain dopamine transmission, as evidenced by a decrease in opioid-evoked dopamine release in the ventral tegmental area, suggesting that pain impairs reward-related circuit in the brain.[56] Besides, morphine administration produces analgesia as well as reward effects; the NAC and the PFC potentially activate after acute or chronic morphine administration and these regions are involved in pain and reward paths.[12] Changes in dopaminergic neuronal activity leads to electrical alterations in the NAC and the PFC,[24] which could adapt to chronic morphine

administration.[14] Morphine-abstinent rats had a low level of neuronal discharging and enhanced level of opioid receptors.[34,54] Sarkaki and coworkers[49] reported that offspring of morphine-dependent parents had less electrical excitability in memory-associated neuronal pathways. Also, structural and physiological changes, as well as reduction of hippocampal long-term potentiation in the litters of morphine-abstinent parents, have been reported.[10,49] We confirmed the findings of Sarkaki et al[49] and observed that the PFC and the NAC neuronal discharging activities was significantly reduced in offspring of morphine-abstinent parents and this reduction was more significant when both parents were morphine-abstinent.

For affirming behavioral and electrophysiological data, we measured opioid receptors (μ, κ, and δ) mRNA levels in the PFC and NAC. Results are in agreement with the observations of Vassoler et al, who showed that adolescent morphine exposure leads to increased μ opioid receptor expression in the NAC of offspring.[58] We found the same results in offspring of the morphine-abstinent parent(s).

It proved that different opioid receptor activations have different effects on pain modalities. A study showed that increased expression of μ opioid receptor had a positive correlation with thermal nociception in the hot plate test.[62] κ Opioid-mutant mice had an elevated response to visceral pain induced by I.P. injection of acetic acid.[20] We propose that upregulation of the κ opioid receptor increased the threshold of visceral nociception in the litters of the morphine-abstinent parent(s). The δ opioid receptor had a major role in mechanical, neuropathic, and inflammatory nociception[37] and was upregulated in the NAC of the morphine-abstinent parents. Morphine administration varies δ opioid receptor expression time-dependently.[22] Therefore, further studies should be undertaken to determine when and how morphine regulates such transgenerational effects in δ opioid receptor expression in offspring of morphine-abstinent parents. Enhancement in the dopamine level also leads to reducing nociception in the litters,[26] and 1 survey in our laboratory showed that the dopamine receptor was upregulated in the litters of morphine-abstinent parents (unpublished research, Zarrindast, et al, 2017).

Opioid receptors' activations initiate ERK1/2 and CREB intracellular signaling during acute and chronic pain.[2,66] During neuropathic pain, the μ opioid receptor/ERK/CREB pathway activated as a self-repair mechanism to stimulate pain-related gene transcription.[65] Probably, the antinociceptive intracellular mechanism of μ opioid receptors in the NAC mediated via ERK/CREB/brain-derived neurotrophic factor pathways in a morphine dependence model,[64] and our study revealed the same results in the offspring of morphine-abstinent parents. Mechanistically, the level of G9a, a repressor of gene expression, in the NAC, was reduced during chronic morphine exposure[51,55] and, consequently, increased CREB/ERK phosphorylation.[35] We hypothesize that the enhancement of μ opioid receptors initiates CREB phosphorylation in the NAC, which subsequently decreases nociception in offspring with morphine-exposed parent(s). On the contrary, we observed no significant changes in the μ opioid receptor mRNA level in PFC. Thus, the role of μ opioid receptor expression level in the NAC of litters of morphine-abstinent rats is involved in the observed change in nociception.

We observed few differences in μ, κ, and δ opioid receptor expression, nociception, and neuronal discharging between the M.ME and P.ME groups. The psychological and molecular reports of addicted/abstinent parents' offspring indicated the equal role of each parent in epigenetic inheritance such as a tendency to drinking, aggregation, drug abuse, etc.[33,49,53] However, experimental animal studies signify the important role of maternal epigenetic inheritance, especially in mammals.[19,29,59]

Conclusions

Our study shows that opiate exposure decreased thermal and visceral pain sensitivities in offspring of morphine-exposed parents. Also, injection of noneffective dose of morphine into these offspring in the formalin test showed a reduction in pain sensitivity. Expression of opioid receptor mRNA was increased in the NAC rather than PFC, which suggests NAC is more sensitive to morphine addiction. Moreover, NAC opioid receptors regulated CREB and ERK1/2 phosphorylation in offspring of the morphine-abstinent parent(s). Neuronal simultaneous firing rates were reduced in the litter of morphine-abstinent parent(s). Consistent with this study and previous reports, these alterations may have long-term consequences in the next progeny. Current data confirm the possibility of endogenous opioid analgesia alternation and the role of heritage in rats with morphine exposed-parent(s).

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